Microbial Source Tracking : Library Independent Host-Specific *Bacteroidales* 16S rRNA Gene PCR Assay in a Mixed Use Watershed

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Abstract

Transport of human and animal wastes into natural waters can result in contamination with fecal pathogens that are increasingly becoming a serious health risk. However, difficulties of differentiating sources of microbial pollutants limit the options to control the pollution. The goal of Microbial Source Tracking (MST) is to identify the source of microbial contamination in natural waters. My MST study was conducted using samples collected from The Upper Sugar Creek Watershed in Ohio. Samples were assayed using a PCR-based molecular method to detect and quantify the *Bacteroidales* 16S rRNA gene. The Sugar Creek Watershed is a mixed-use watershed suitable to examine the source of microbial contamination from human and agricultural activity and/or wildlife. Host-specific *Bacteroidales* assays (human, ruminant, horse, pig, and dog) were used to determine potential host sources. A quantitative PCR (qPCR) assay for general *Bacteroidales* was used to investigate the magnitude of fecal contamination. Viable counts of *E.coli* were determined for statistical comparison with the *Bacteroidales* PCR assay. These data were analyzed along with land management data. We found frequent human specific signals at residential land use areas and also observed high magnitude of general *Bacteroidales* qPCR signals at concentrated livestock operation areas and in residential areas. The results indicate the potential application of the MST method to aid in making land management decisions to control microbial contamination at the watershed scale.

Introduction

Understanding the source of potential pathogens in natural waterways is important in order to predict risks to human health. Also it is necessary to develop land management strategies for protecting water resources. However, the source of microbial contamination for most waterborne disease outbreaks cannot always be identified. Source identification is required to monitor the responsibility to avoid conflict among public health agencies, farmers, industries, and local residents. MST has been studied by using indicator bacteria, either culture-based methods or molecular methods. The development of molecular methods give us an advantage for the source tracking in a watershed level because of its easiness compared to time consuming culture-based methods which requires culturing process and library developing. The purpose of this study was to determine the applicability of this MST method in a specific geological area and to find the relationship between microbial contamination and land use practices.

Study Site

In 2000 the Ohio Environmental Protection Agency (EPA) labeled the Sugar Creek Watershed as the second most impaired in Ohio. The Sugar Creek watershed contributes to the hypoxia in the Gulf of Mexico because it is located in the headwaters of the Muskingum Watershed, Ohio's largest watershed flowing to the Ohio River. The Upper Sugar Creek Watershed (Fig.1) has different land uses with potential contaminant sources such as residential areas, crop fields, livestock operations (dairy, sheep, horse, and swine), and natural forested areas. This mixed-use watershed is suitable to examine the source of microbial contamination from human and agricultural activity and/or wildlife.

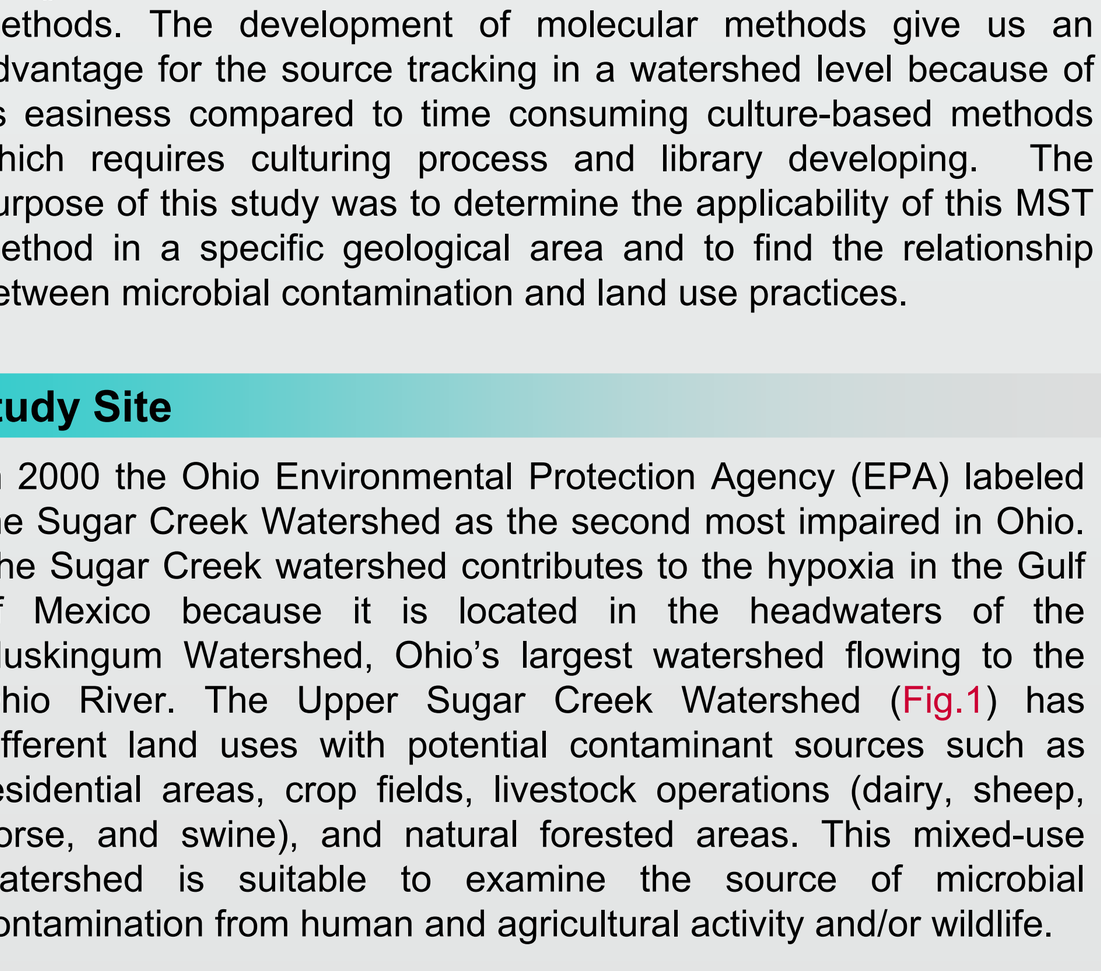

Figure 1. Upper Sugar Creek Watershed mapped with land use data (NLCD 2001, USDA), A total of 21 sampling points in sub-watersheds are identified.

Materials and Methods

►**Environmental water sample collection and E. coli measurements:** Stream water samples were collected three different times from 21 sampling sites in the summer of 2008. Water samples were placed in a cooler, taken to the laboratory and processed within 6 hours. Viable *E. coli* counts were obtained using the Colilert ® Method with Quanti-Tray/2000™ (IDEXX,ME).

Materials and Methods (continued)

►**Sample filtration, DNA extraction and PCR amplification:** Water samples (100 ml) were filtered (0.2 µm filter) to collect bacterial cells and then the DNA was extracted using the PowerSoil DNA Kit (MoBio, CA). PCR reactions were conducted using general primers to confirm the presence of *Bacteroiodales* in the samples.

►**Host specific PCR:** Ruminant- and human-specific *Bacteroidales* 16S rRNA gene markers were tested for host specificity and then used for host specific *Bacteroidales* detection in water samples. All forward primers specific for each marker (CF128f and CF183f for ruminant specificity and HF134f and HF183f for human specificity) were paired with the general reverse primer, Bac708r. Ten individual cow feces, seven individual human feces, one water-treatment plant influent and one septic influent were used to test host specificity. Primer specificity was further investigated using pooled fecal DNA samples (pig, deer, sheep, dog, goose, and horse).

►**Quantitative PCR (qPCR):** A general set of primers that targeted the 16S rRNA gene of *Bacteroidales* markers were used to assess the magnitude of concentrations in the samples. For each qPCR run, all samples were analyzed in triplicate. PCR inhibitors in the samples were determined to be negligible based upon results obtained after 10-fold and 100-fold dilutions.

Results - General *Bacteroidales* qPCR

All samples except Sample Point 8* contained a detectable amount of *Bacteroidales* marker (see Fig. 1 for the location of each sample site) with concentrations ranging from 10^5 to 10^9 DNA target segments per 100 ml sample. There was variation in marker concentrations among the three sampling events (Fig. 2). Recurrent high quantities were observed at Sample Points 14 and 21 (indicated with red circles in Fig. 2). This suggests a possible association between fecal contamination and land use. The sub-watershed of sampling point 14 had a residential area right above the sampling spot. Sample Point 21 had a pasture for dairy cows and tile lines from this pasture contributed to the water at this sample point.

*(Sample Point 8 was a community-maintained natural spring used as a drinking water supply.)

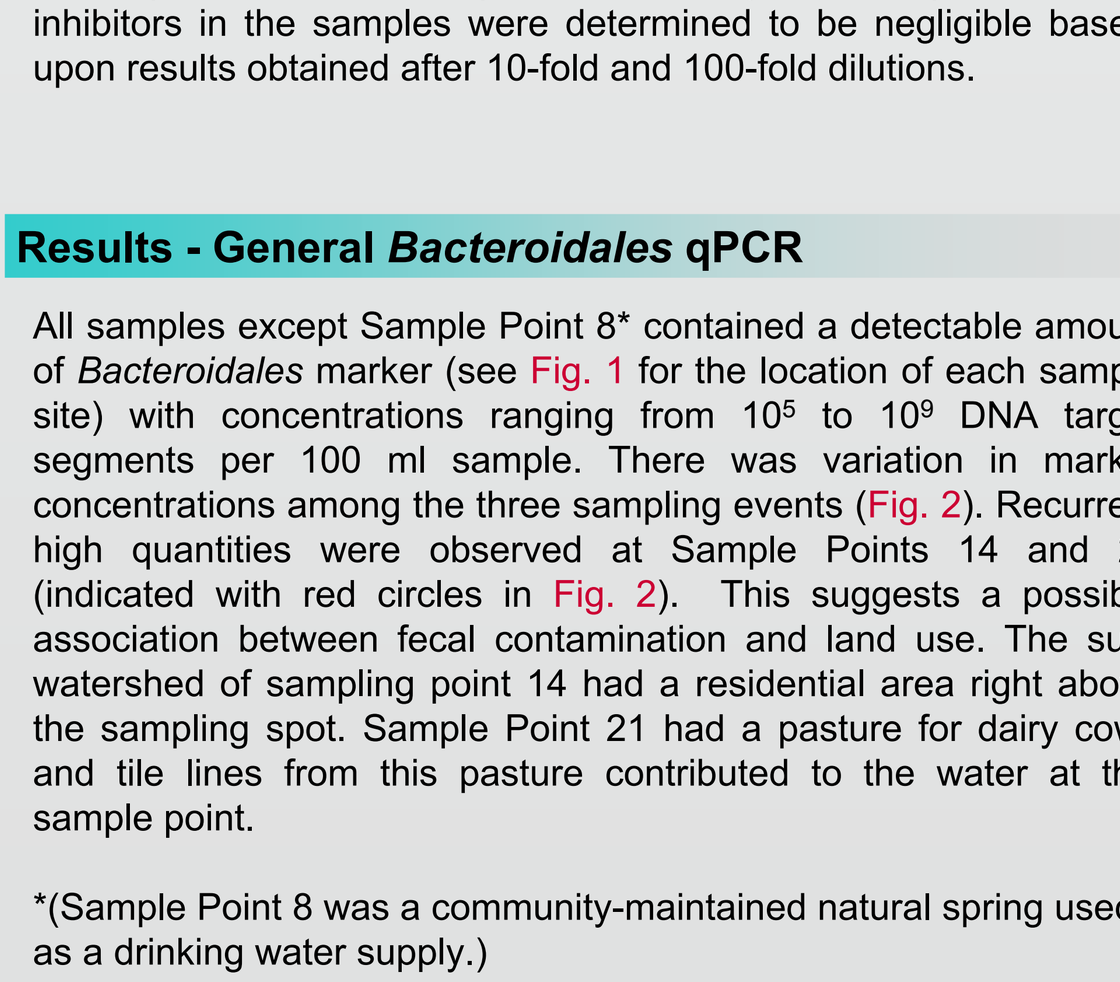

Figure 2. qPCR results with general *Bacteroidales* 16S rRNA gene markers. Values are geometric mean of three samplings and error bar indicates one standard error.

Results - Host Specificity and Sensitivity Study

Ruminant- and human-specific *Bacteroidales* 16S rRNA gene markers were tested for specificity and sensitivity. The two ruminant specific markers displayed 100% sensitivity for individual cow feces DNA. However, they also tested positive for other ruminant species (deer and sheep). In addition, one of the markers (CF128f) was positive for pig feces DNA (Fig. 3). The two human specific markers displayed 86% sensitivity towards individual human feces DNA and were positive for both wastewater influent DNA, and septic influent DNA, considered to be pools of human fecal DNA. No false positives were observed with other host DNA pools (Fig. 4).

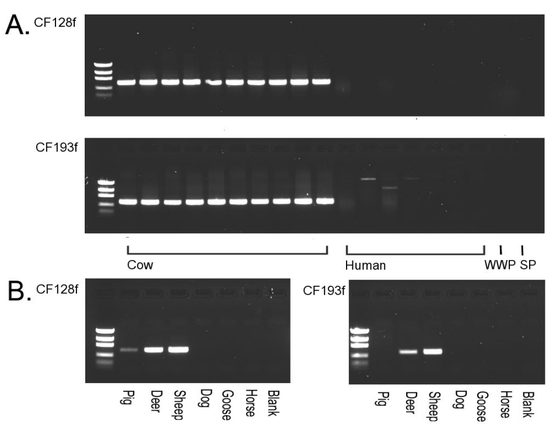

Figure 3. Host specificity PCR results with two ruminant specific primers (CF128f, CF183f).
(A) ruminant specific primer distinguished cow fecal DNA from human fecal DNA, wastewater influent DNA (WWP), and septic influent DNA (SP).
(B) Deer and sheep fecal DNA were also positive for both markers and pg fecal DNA was positive for CF128f.

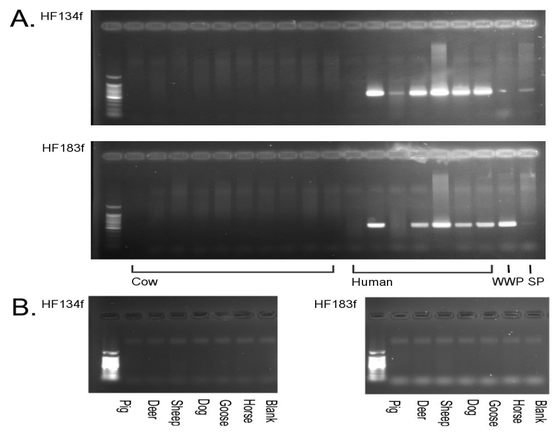

Figure 4. Host specificity PCR results with two human specific primers (HF134f and HF183f).
(A) False positive on cow fecal DNA was not observed. One out of 7 individual human fecal samples was falsly negative for both primers. Wastewater influent DNA (WWP), and septic influent DNA (SP) both tested positively.
(B) No false positives were observed on other host DNA samples.

Result - Correlation between *Bacteroidales* qPCR method and Viable *E.coli* count

Viable counts of *E.coli* were determined for statistical comparison with the *Bacteroidales* PCR assay (Fig.5).
Non Parametric Spearman's rank correlation coefficient was calculated based on the collected data pairs (n=59). There was a statistically moderate positive correlation (Spearman's rank correlation coefficient:0.545, P<0.001) observed between *E.coli* and *Bacteroidales* quantity in water samples.

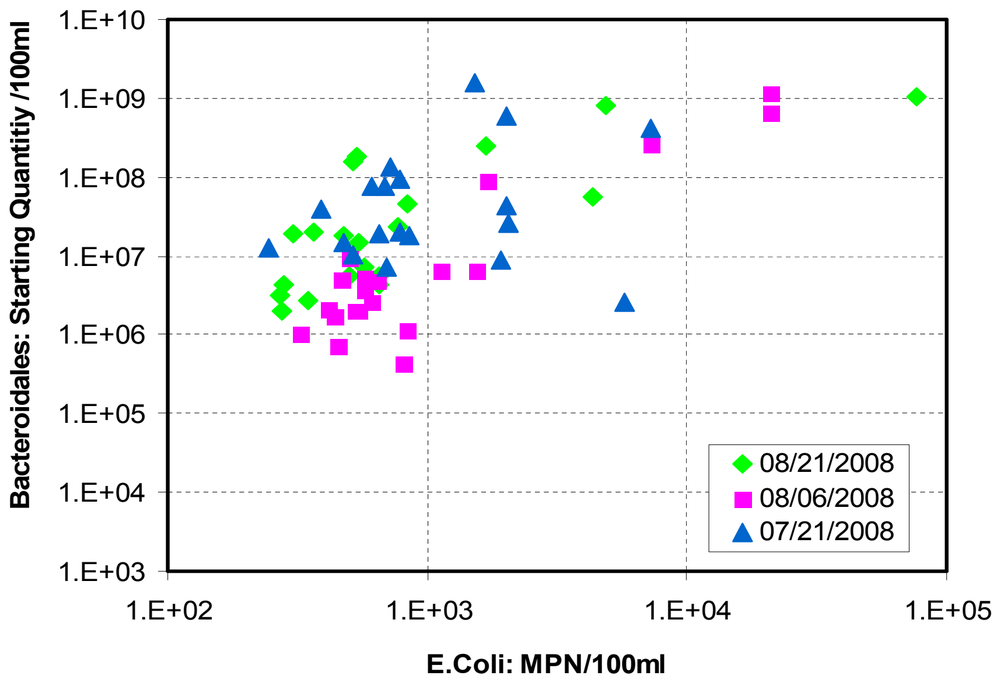

Figure 5. Scatter plot showing the relationship between *E. coli* Most Probable Number and *Bacteroidales* qPCR results.

Spearman's rank correlation coefficient was 0.545 (n=59, P<0.001).

Results - Host Specific PCR

Ruminant- and human host-specific PCR were conducted on all stream water samples. Two ruminant markers were strongly positive in samples from Sample Point 21 on July 21 and weakly positive in samples from Sample Points 5, 6, and 18 on August 6. Human host-specific PCR positives were also observed in water samples from various sites including the Sample Points 14 and 21 where an intense general *Bacteroidales* qPCR signal was observed (Table 1).

	Human Markers						Ruminant Markers					
	HF134f			HF183f			CF128f			CF193f		
Site	21-Jul	6-Aug	20-Aug	21-Jul	6-Aug	20-Aug	21-Jul	6-Aug	20-Aug	21-Jul	6-Aug	20-Aug
1		+	+									
2	+											
3	+	+	+	+		+						
5	+			+				+			+	
6		+			+			+			+	
9	+			+								
11	+			+								
12			+									
14	++	++	++	++	++	++						
15	+			+								
17			+	+								
18	++	+	++	++	++	+					+	
19	++											
20	++											
21	++	++	++	++	++		++			++		

Table 1. Host Specific PCR result for all samples. No host-specific positive found at site 4, 7, 8, 10, 13, and 16. **+**: Weak positive **++**: Strong positive. The intensity of these positive signals were judged by comparison to reference positive controls on electrophoresis gel image.

Conclusion

***E. coli* is a culturable aerobic bacterium and is widely used as fecal indicator to regulate water quality. *Bacteroidales*, the subject of this study, is an anaerobic bacterium and is expected to have limited survival after release into the environment. We observed a positive correlation between *E. coli* and *Bacteroidales* numbers in environmental water samples. However, we believe *Bacteroidales* is a more sensitive and specific indicator of fecal contamination.**

Host specific PCR analyses revealed the origin of *Bacteroidales* with the human specific marker clearly able to distinguish human fecal contamination from agricultural / wildlife fecal contamination. Ruminant specific analysis was not able to differentiate between cow and deer. However, a combination of host specific PCR analyses and land use data analyses will make the microbial source tracking method more accurate and a powerful tool in making land management decisions that affect microbial contamination at the watershed scale.

Supporting References

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Acknowledgements

This work was supported by the Water and Watersheds Program of the National Research Initiative (USDA-CSREES). The project title is "Upper Sugar Creek Watershed, Ohio: A Model Watershed for Study of Pathogen Origin, Fate and Transport". Support has also been provided in the form of facilities and equipment by The Ohio State University-Ohio Agricultural Research and Development Center, Wooster, OH and The Ohio State University School of Environment and Natural Resources, Columbus, OH.